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Extracellular proteins limit the dispersal of biogenic nanoparticles

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1 **Summary:** Proteins are trapped within dense aggregates of bacterially-formed metal
2 sulfide nanoparticles, and may have played a key role in the aggregation process.

3
4 **Micron-scale, concentrically-zoned spheroidal aggregates of biogenic zinc sulfide**
5 **nanoparticles formed by sulfate-reducing bacteria in natural biofilms contain**
6 **polypeptides or proteins. The previously unreported intimate association of**
7 **peptide-rich organic matter with metal-sulfides is a form of extracellular**
8 **biomineralization. Experiments involving synthetic ≤ 3 nm-diameter ZnS particles**
9 **and a suite of representative amino acids indicate an important role for cysteine in**
10 **driving rapid particle aggregation. The findings suggest that microbially-derived**
11 **proteins can limit the dispersal of abundant nanometer-scale bioremediation**
12 **products that otherwise may be transported by fluid flow in the subsurface.**

13

14 **Introduction**

15 Sulfate-reducing bacteria (SRB) can lower the concentrations of metals in anoxic waters
16 by sequestering metals into nanoparticles (e.g., 1-3). However, these particles are
17 potentially highly mobile due to their small size (4). The smallest particles are < 2 nm
18 diameter, comparable to molecular clusters (6), while most are 2 to 6 nm (2, 5). Small
19 size facilitates both transport and oxidative re-dissolution (7, 8). Aggregation can restrict
20 transport by inducing settling of colloids from solution (9, 10), simultaneously promoting
21 crystal growth and decreasing solubility (11, 12). Amine-bearing organic molecules have
22 been shown to organize sulfide nanoparticles into semiconductor nanowires (13). In this
23 study, we investigated the hypothesis that natural organic matter contributed to the

1 formation of densely-aggregated nanoparticulate ZnS spheroids, and remains preserved in
2 nanometer-scale pores (5). We use microanalytical and direct isolation approaches to
3 analyze nanoparticle aggregates formed in natural sulfate-reducing bacterial biofilms
4 (14), and experimentally evaluate the potential for amino acids to induce the rapid
5 aggregation of metal-sulfide nanoparticles.

6 We examined natural SRB-dominated biofilms collected from the Piquette Pb, Zn
7 mine in southwestern Wisconsin, USA (14). Ultramicrotomed sections of biofilm
8 containing spheroidal aggregates of biogenic ZnS nanoparticles (Figs. S1-S3) were
9 imaged with transmission and scanning electron microscopy (TEM and SEM,
10 respectively) prior to *in situ* isotopic microanalysis with high-resolution secondary ion
11 mass spectrometry (NanoSIMS) at a spatial resolution of ~50 nm (15).

12 Comparison of TEM/SEM images with NanoSIMS ^{32}S isotope maps demonstrates
13 that ZnS spheroids are the only structures within the biofilm that contain significant
14 concentrations of sulfur. The composite NanoSIMS data show the intimate association of
15 $^{12}\text{C}^{14}\text{N}$ with biofilm ZnS (Figs. 1, S7, S8) at significantly higher levels than in the abiotic
16 ZnS reference materials (Fig. S5). Nitrogen is present throughout the biofilm ZnS
17 aggregates. Pores in the ZnS aggregates appear as low diffraction-contrast features in
18 TEM images, due to a lower concentration of sphalerite nanoparticles (Fig. S2). These
19 porous regions are associated with the highest $^{12}\text{C}^{14}\text{N}$ intensities (N concentrations; Figs.
20 S7, S8). In comparison, composite isotope maps of reference materials show that the
21 nitrogen concentrations in synthetic ZnS aggregates are ~100x lower than in biofilm ZnS
22 (Fig. S5). Based on an empirical relative sensitivity factor for N in ZnS, we estimate

1 from analysis of 134 ZnS spheroids an average nitrogen concentration of 1.60 wt% ±
2 0.22 (s.d.).

3 Nitrogen in the biofilm ZnS could be present as either an organic or inorganic
4 form. Dissolved nitrate is present in mine waters at concentrations ~3 ppm, but this small
5 amount should be removed from the biofilm during sample processing. We tested this
6 hypothesis by analyzing the biofilm for $^{14}\text{N}^{16}\text{O}$, which yielded very low counts relative to
7 $^{12}\text{C}^{14}\text{N}$ (15), indicating that N is not present as an oxidized species (e.g. nitrate, nitrite).
8 The $^{12}\text{C}^{14}\text{N}/^{14}\text{N}^{16}\text{O}^-$ ratio in the biofilm and ZnS spheroids was compared to this ratio in a
9 sample of KNO_3 in graphite as a nitrate reference (USGS-32 KNO_3 in graphite) and to
10 bacterial spores as an organic nitrogen reference, to test for significant levels of nitrate in
11 the ZnS spheroids. The $^{12}\text{C}^{14}\text{N}/^{14}\text{N}^{16}\text{O}^-$ ratio in the KNO_3 in graphite sample ranged from
12 1 to 150, with a weighted average of ~15. The $^{12}\text{C}^{14}\text{N}/^{14}\text{N}^{16}\text{O}^-$ ratio in the bacterial spores
13 ranged from 2000 to 4000. The $^{12}\text{C}^{14}\text{N}/^{14}\text{N}^{16}\text{O}^-$ ratio in the biofilm and biofilm ZnS is
14 2000 to 3500. Based on these analyses, we conclude that nitrogen in the biofilm is not
15 associated with oxygen and is organic in nature. This conclusion is further supported by
16 the presence of amide absorption features in the infrared spectroscopy data below. We
17 note that, from the N content of ZnS estimated above, and using an average amino acid N
18 concentration ~11 wt%, the ZnS spheroids could contain as much as ~14 wt% amino
19 acids.

20 Areas enriched in $^{12}\text{C}^{14}\text{N}$ with cell-like morphologies (Fig. 1) and increased ^{31}P
21 content (data not shown) are interpreted as either whole or degraded microbial cells.
22 These features are morphologically distinct compared to ZnS spheroids, arguing against
23 spheroid formation by encrustation of microbes. We thus infer that the ZnS spheroids

1 formed by aggregation of ZnS nanoparticles generated by BSR with polypeptides or
2 proteins. The process may have involved ZnS nanoparticles with surface adsorbed
3 peptides or co-aggregation of protein molecules and nanoparticles.

4 Synchrotron-based Fourier-transform infrared spectroscopy (SR-FTIR), with ~10
5 μm spatial resolution, was used to further classify organic molecules detected within
6 aggregates (15). SR-FTIR analyses revealed that absorptions at both $\sim 1,580\text{ cm}^{-1}$ and
7 $\sim 1,640\text{ cm}^{-1}$ are only associated with ZnS spheroid-rich regions of the biofilm (Fig. 2).
8 Absorption features at $\sim 1,580\text{ cm}^{-1}$ and $\sim 1,640\text{ cm}^{-1}$ are assigned to amide II and amide I
9 vibration modes, respectively, and are characteristic of polypeptide- and/or protein-
10 derived amino acids (17). Analyses varied in the relative magnitudes of amide I and II
11 absorption features by only a few percent. The SR-FTIR data confirm that $^{12}\text{C}^{14}\text{N}$
12 measured in NanoSIMS analyses of spheroid-rich regions of biofilm originate from
13 polypeptides or proteins, and suggest that these regions contain increased concentrations
14 of these components relative to spheroid-free regions.

15 Proteins were directly extracted (15) from density-separated fractions dominated
16 by either organic biofilm components or ZnS spheroids (Fig. S6). Proteins from the
17 biofilm fraction produce faint bands in 4-10% polyacrylamide gradient SDS gels at
18 molecular weights of $\sim 37\text{ kDa}$ and $\sim 48\text{ kDa}$ (15; Fig. 3). In contrast, the ZnS-enriched
19 fraction yielded a more predominant band at $\sim 37\text{ kDa}$, suggesting that the amino acids
20 detected by NanoSIMS and SR-FTIR are associated with protein(s) of this molecular
21 mass. It was not possible to identify this protein or group of proteins or to assess the
22 degree of functionality or degradation state, due to the limited availability and
23 accessibility (14) of sample. However, a size of 30 – 50 kDa is consistent with known

1 bacterial metal-binding proteins functionally similar to metallothionein (e.g., 17-19).
2 Recently, the SRB species *Desulfovibrio desulfuricans* and *Desulfococcus multivorans*,
3 grown in media with Cd^{2+} , were shown to express genes homologous to *SmtA* (20), a
4 gene coding for a protein required by *Synechococcus* for Zn^{2+} resistance (21). An
5 unidentified extracellular metal-binding compound has also been partially purified from
6 *D. multivorans* (22), a member of the family *Desulfobacteraceae*, one of two major SRB
7 groups active within the biofilm (14). These similarities raise the interesting possibility
8 that the ZnS-associated protein(s) found in this study may serve a similar function.

9 The low-density zones between concentric shells of densely packed ZnS
10 nanoparticles (5) likely accommodate larger proteins and may represent peptide-rich
11 organic-coatings that were adsorbed to aggregate surfaces between episodes of ZnS
12 nanoparticle precipitation (e.g., resulting from seasonally controlled cycles of SRB
13 activity). The high $^{12}\text{C}^{14}\text{N}$ regions surrounding the outermost surfaces of some aggregates
14 may be protein-rich coatings that are the contemporary equivalent. Alternating bands of
15 organics and ZnS may record temporal patterns of microbial activity.

16 In some aggregates NanoSIMS data indicate overlapping $^{12}\text{C}^{14}\text{N}$ and ^{32}S
17 distributions, implying fine-scale mixtures of ZnS nanoparticles and protein-rich organic
18 matter. Thus, a fraction of the amino acids may be bound to the surfaces of
19 nanoparticles. Known bacterial metal-binding proteins bind zinc and other potentially
20 toxic metals (e.g., Cd, Cu), primarily at cysteine residues in proximity to OH^- groups
21 (23). Experimental evidence shows that (1) cysteine also binds strongly to ZnS
22 nanoparticles and limits their size to $< \sim 5$ nm (24), and (2) thiol groups bind strongly
23 with sulfur-deficient surface Fe(II) atoms in pyrite (FeS_2) (25). The conditional stability

1 constant for mono-ligand cysteine-Zn²⁺ complexation in low ionic strength solutions (\leq
2 0.1 M_c) at 20-25 °C is $\gt 4$ orders of magnitude larger than those of all the other amino
3 acids tested except for lysine, for which the constant is ~ 2 orders of magnitude larger
4 (15). These observations suggest that cysteine or cysteine-rich polypeptides or metal-
5 binding proteins could have played a role in determining the ZnS particle size and
6 aggregation state.

7 We tested the efficacy of individual amino acids (100 μ M of alanine, aspartate,
8 cysteine, lysine, phenylalanine, proline, and serine) to promote aggregation of synthetic <
9 3 nm-sized ZnS (10 μ M ZnS) (15). Aggregation was monitored periodically using
10 dynamic light scattering (DLS; 15). Results showed that the inorganic aggregation of
11 ZnS occurred rapidly at first, to form ~ 100 nm-diameter aggregates, but then slowed
12 greatly or ceased after one week (Figs. 4, S10). The dynamic light scattering for ZnS
13 after one week of continuous reaction was $\sim 5 \times 10^4$ counts per second (cps). In contrast,
14 cysteine plus ZnS yielded $\sim 8 \times 10^4$ cps, nearly twice that of ZnS alone, and a direct
15 indication of the presence of larger aggregates in solution. Cysteine also prolonged the
16 aggregation process; after one week the average aggregate size continued to increase,
17 ultimately forming 1-10 μ m-sized structures. Other amino acids had little (e.g., serine,
18 lysine) to no (e.g., alanine, phenylalanine) detectable effect on nanoparticle aggregation.
19 Cysteine in the absence of ZnS produced $\sim 3 \times 10^4$ cps and formed no measurable
20 aggregates (data not shown). Humic compounds added to ZnS nanoparticle suspensions
21 did not accelerate aggregation. In conclusion, the effect of cysteine on ZnS nanoparticle
22 aggregation rate and final aggregate size is pronounced relative to that of other amino

1 acids or ZnS nanoparticles alone. Our experimental results suggest a role for cysteine or
2 cysteine-bearing molecules in biofilm ZnS spheroid formation.

3 Mineral-protein mixtures with internal organization are typically considered
4 biominerals, and biominerals normally form within organisms. The structures reported
5 here represent an exception to this pattern. Proteins, peptides, and amino acids could be
6 released after cell death and scavenged by hydrophobic ZnS surfaces. Alternatively,
7 bacteria may export Zn-binding proteins for a physiological reason. Most known
8 bacterial metal-binding proteins are produced for intracellular binding and subsequent
9 export of toxic metals (23). In the case reported here, exported metal-binding proteins
10 may promote aggregation-induced “clearing” (26) of very small and potentially toxic (27)
11 metal-sulfide nanoparticles from the biofilm solution, preventing incidental uptake into
12 cells (e.g., 28) and/or cell entombment. Hydrophobic interactions among peptide/protein-
13 ZnS complexes may have induced formation of dense (rather than open, fractal-like)
14 aggregates, and led to the observed spheroidal morphology. Densely packed aggregates
15 of metal-bearing nanoparticles, similar to the biofilm ZnS spheroids characterized in this
16 study, have been reported in other metals-contaminated systems (e.g., 39). Similar
17 aggregates may be present in sediments, where they could trap and preserve organic
18 molecules.

19 There is a growing body of evidence for both nanoparticle-based contaminant
20 transport (1-5) and trapping of nanoparticles in the vicinity of their formation site (30).
21 Our results suggest that metal-binding polypeptides and proteins play a key, and
22 previously unrecognized, role in extracellular metal-sulfide biomineralization, and may
23 be an important factor limiting nanoparticle dispersal in natural environments.

1

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3

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Figure Captions:

Figure 1: NanoSIMS secondary ion images showing ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S in ultramicrotomed TEM section of biofilm and spheroidal ZnS aggregates. (A) Composite isotope map of ^{12}C (blue), $^{12}\text{C}^{14}\text{N}$ (green), and ^{32}S (red) for $\sim 10\ \mu\text{m} \times 10\ \mu\text{m}$ region of biofilm (area “C2.9”). Secondary and tertiary colors vary in hue and intensity to reflect relative proportions of two or three isotopic components, respectively. (B) Box key to color-coded isotopic distributions seen in (A); e.g. in (A), uniformly red spherules are relatively pure ZnS, while orange or yellow halos surrounding some spherules indicate enhanced levels of nitrogen. The key is projected through the ternary end member that would represent a combination of maximum ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S counts (i.e. through “white”). (C) SEM image of biofilm and ZnS spheroids in part of the region shown in (A). White arrows point to the same individual spheroids in (A) and (C) for reference. Scale bars = $1\ \mu\text{m}$.

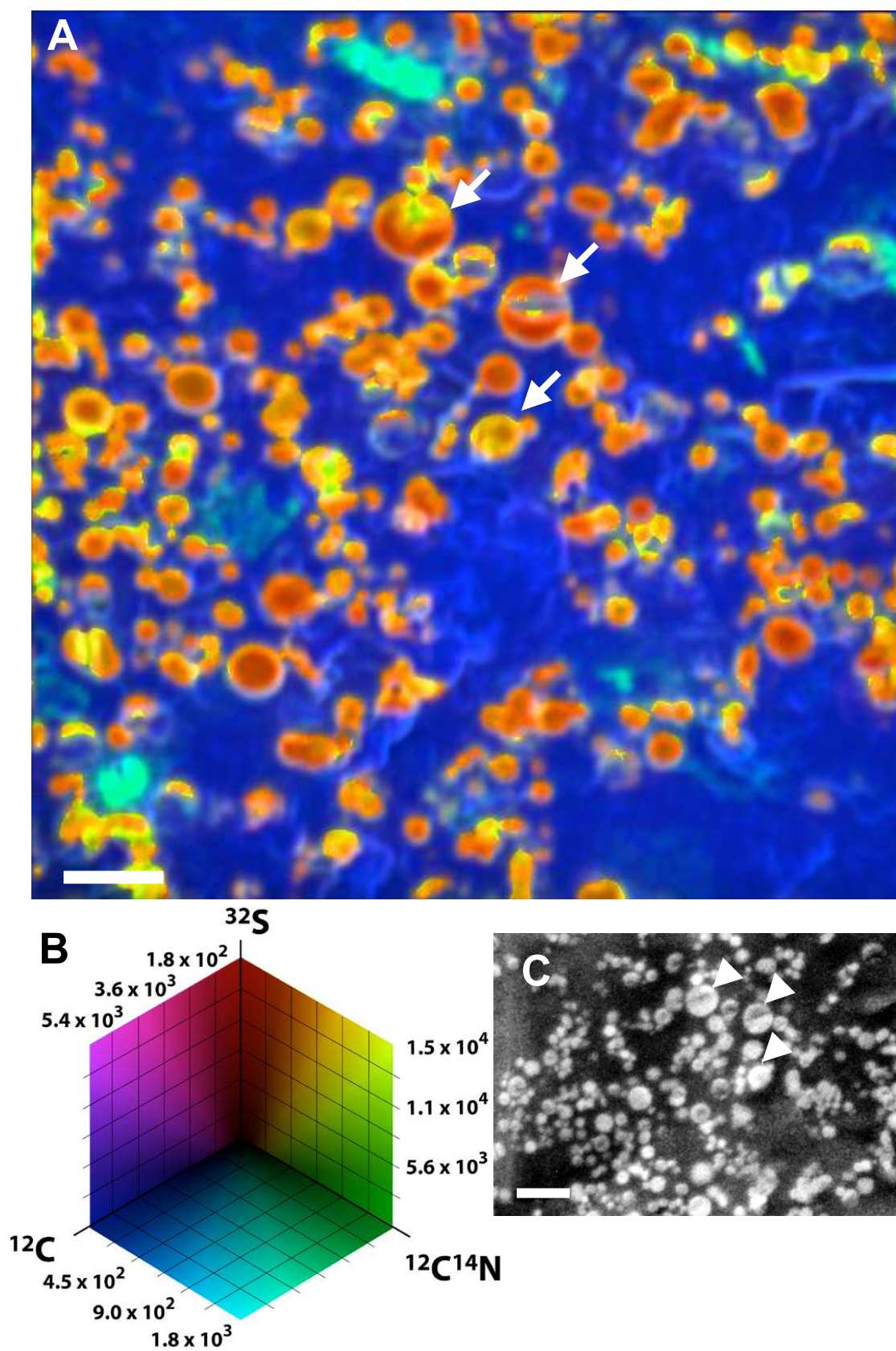
Figure 2. SR-FTIR transmission spectra of biogenic ZnS aggregates (black) and background biofilm (grey). Amide I ($\sim 1640\ \text{cm}^{-1}$) and II ($\sim 1580\ \text{cm}^{-1}$) absorption features are diagnostic of amino-acid associated bond vibrations in polypeptides and/or proteins.

Figure 3: Composite SDS-PAGE gel electrophoresis image of biofilm and ZnS protein extractions. SyproOrange and colloidal silver molecular weight standards (far left and right lanes, respectively). (A) Extraction from the biofilm organic fraction

1 stained with SyproOrange. **(B)** Extraction of the ZnS spheroid fraction stained with
2 SyproOrange. **(C and D)** Replicate extractions of biofilm organic fraction stained with
3 silver. **(E and F)** Replicate extractions of ZnS spheroids fraction stained with silver.
4 Numbers are molecular masses in kDa.

5
6 **Figure 4: Size distribution curves from dynamic light scattering (DLS) data**
7 **acquired in ZnS nanoparticles aggregation experiments.** **(A)** Control experiments. 10
8 μM ZnS nanoparticles alone (solid lines) aggregate within 1 day to form ~ 100 nm radius
9 clusters that exhibit little further growth over the 5 day period. 100 μM cysteine alone
10 (dashed lines) gives a very weak DLS signal with is no consistent trend in size
11 distribution. **(B)** In the presence of both 10 μM ZnS and 100 μM cysteine, sustained
12 aggregation occurs over the 7 day period, resulting in aggregates that are more than 1
13 order of magnitude larger than the initial clusters. DLS correlation functions from which
14 size distributions were derived are shown in Fig. S9.

1 Figure 1



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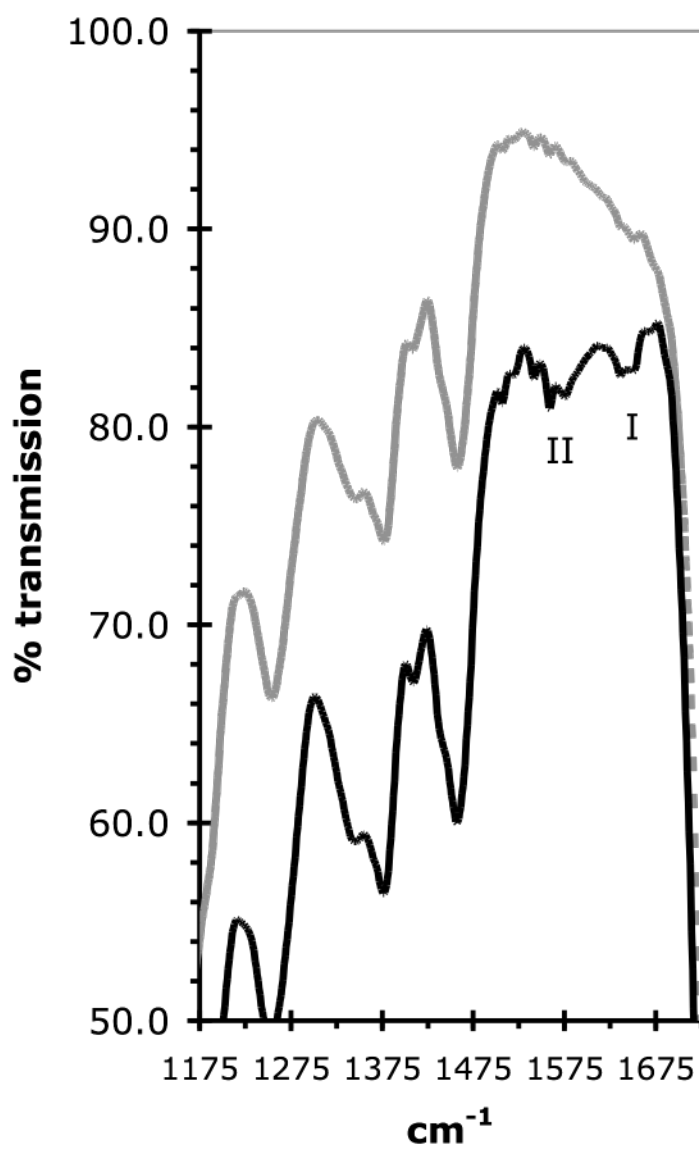
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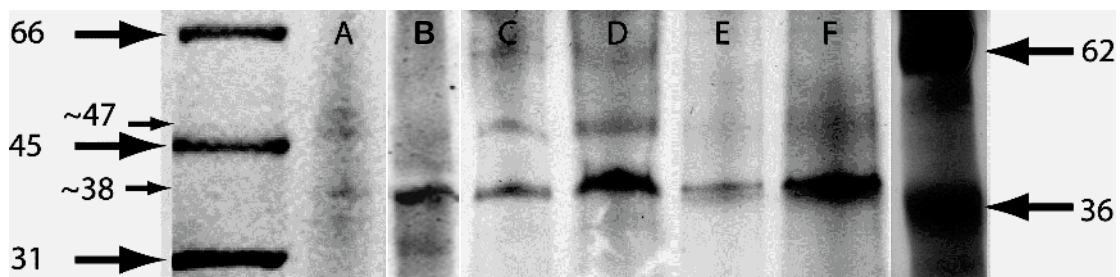
Figure 2

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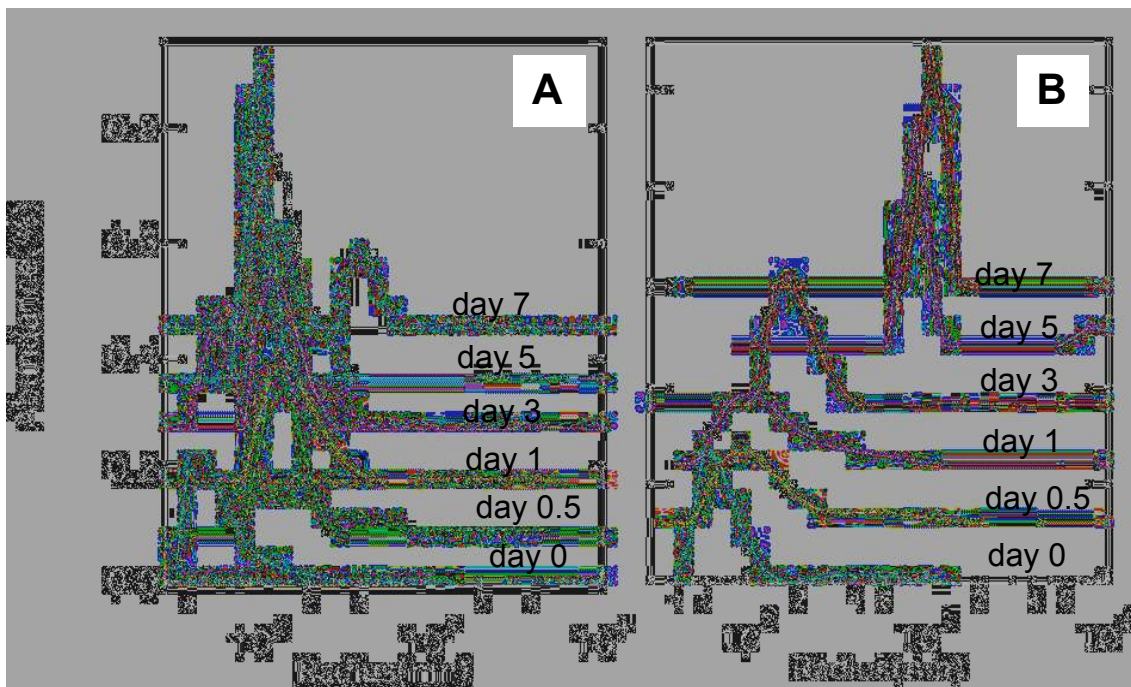
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Figure 4

1 **Supplementary Materials and Methods:**

3 *TEM/NanoSIMS biofilm sample preparation:*

5 Samples of biofilm were fixed with 4% glutaraldehyde, as described previously (1).
6 TEM images (Fig. S4) confirm that, because the sectioning resin neither displaces nor
7 damages fine-scale biofilm features, ultramicrotomed samples of biofilm used for TEM
8 imaging were also suitable for NanoSIMS analyses.

10 *Secondary Ion Mass Spectrometry (SIMS):*

12 SIMS was performed using the Lawrence Livermore National Laboratory NanoSIMS 50
13 (Cameca, Gennevilliers Cedex, France). The measurements were made with a 0.2 to 0.7
14 pA, 16 keV $^{133}\text{Cs}^+$ primary ion beam focused into a 50 to 100 nm diameter spot, rastered
15 over sample areas of $\sim 10 \times 10 \mu\text{m}^2$. The isotope imaging measurements consist of 15 to
16 100 replicate scans of 256 x 256 or 512 x 512 pixels with dwell times of 1-2 ms/pixel.
17 Secondary ion intensities were collected simultaneously in multi-collection mode using
18 three different collector configurations, [$^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{31}\text{P}^-$, $^{32}\text{S}^-$, $^{34}\text{S}^-$], [$^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{14}\text{N}^{16}\text{O}^-$,
19 $^{32}\text{S}^-$, $^{14}\text{N}^{32}\text{S}^-$] and [$^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{34}\text{S}^-$, $^{14}\text{N}^{32}\text{S}^-$]. A mass resolving power of ~ 4000 was
20 used. Samples were also simultaneously imaged using secondary electrons. Nitrogen
21 concentration in the ZnS aggregates was estimated based on a relative sensitivity factor
22 for NS^- (RSF_{NS}) in ZnS of $4.2 \times 10^{24} \text{cm}^{-3}$ (2). The atom abundance of N per cm^3 of ZnS
23 (C_{N}) is estimated from:

$$25 \quad \text{C}_{\text{N}} = \text{RSF}_{\text{NS}} * \text{I}_{\text{NS}}/\text{I}_{\text{S}},$$

27 where I_{NS} and I_{S} are the isotope corrected ion count rates for NS^- and S^- (3). The mass
28 abundance of N per mass ZnS is calculated from C_{N} , Avogadro's number, the atomic
29 mass of N and the density of ZnS. $^{12}\text{C}^{14}\text{N}^-$ is used in the presented images because it has a
30 $\sim 100\times$ higher ion yield than $^{14}\text{N}^{32}\text{S}^-$ and therefore provides higher clarity images.
31 Composite multi-isotope image maps were constructed using Photoshop and the
32 NanoSIMS data analysis software, *L'image* (developed by L. Nittler, Carnegie Institution
33 of Washington, Washington, DC, USA). Grey-scale $^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$ and $^{32}\text{S}^-$ intensity images
34 generated by *L'image* were assigned to blue, green and red channels, respectively, in
35 Photoshop RGB image files. Primary colors in accompanying image map keys represent
36 single isotope concentrations, whereas secondary or tertiary colors represent mixtures of
37 two of three isotopic components. Black spots indicate areas of no data (i.e. holes in the
38 TEM section). The significance of measurements obtained via NanoSIMS analyses of
39 biofilm ZnS was determined by comparison to NanoSIMS analyses of natural and
40 synthetic ZnS reference materials (Fig. S5) for $> 1,000$ regions-of-interest (ROIs) of ~ 180
41 nm^2 each. In addition to a natural single-crystal ZnS standard (Balmat sphalerite,
42 NBS123), nanoparticulate ZnS synthesized by H. Zhang (UC Berkeley) was used to
43 represent a similarly structured (i.e., nanocrystalline) pure ZnS reference material (4) for
44 comparison.

45

1 *Synchrotron-radiation Fourier transform infra-red spectroscopy (SR-FTIR):*

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3 SR-FTIR is capable of detecting and differentiating amongst nucleic acids, lipids, amino
4 acids, and polysaccharides (5). The spatial resolution of SR-FTIR, coupled with optical
5 microscopy, is diffraction limited, or between 2 and 10 μm in the mid-infrared (6). SR-
6 FTIR analyses were conducted on uncoated ultramicrotomed sections of the same biofilm
7 samples used for NanoSIMS analyses. The location of the synchrotron probe relative to
8 targets selected with optical microscopy was calibrated using IR-sensitive targets on
9 standards. Background spectra were obtained and used as reference spectra for both
10 samples and standards to remove atmospheric H_2O and CO_2 absorptions.

11
12 *Biofilm/ZnS separation and total protein extraction:*

13
14 Biofilm samples were homogenized by repeated pipetting action through a series of
15 sterile syringe needles of increasingly smaller inner-bore diameter (18-, 21- and 22-
16 gauge, respectively, for approximately 20 minutes each). Homogenized biofilm slurries
17 were then pelleted by centrifugation, re-suspended in lithium polytungstate solution
18 (LST) of density $\sim 2.8 \text{ g cm}^{-3}$ (Geoliquids, Prospect Heights, IL, USA), and then subjected
19 to further low-speed centrifugation (~ 5 mins at 1000 rpm) in autoclaved microcentrifuge
20 tubes (Eppendorf, Hamburg, Germany). ZnS-rich biofilm fragments ($\rho \sim 4.1 \text{ g cm}^{-3}$) were
21 easily pelleted at the bottom of LST-filled tubes during centrifugation, while ZnS-poor
22 biofilm fragments rose to the top of the LST solution to be re-pipetted into new tubes.
23 This process of low-speed centrifugation, density-based fractionation, collection and re-
24 suspension was repeated several times, until no further separation of ZnS-rich and ZnS-
25 poor biofilm fractions was observed. Both fractions were transferred a final time by
26 pipette to clean tubes of nuclease-free water, and then pelleted, washed and re-suspended
27 6-8 times, to remove all traces of LST. Each resulting fraction represents a subsample of
28 biofilm or ZnS spheroids with greatly increased relative concentrations of organic matter
29 or ZnS, respectively. This method avoids the use of surfactants or solvents that might
30 solubilize and destroy organic biomolecules, including nucleic acids and proteins. Each
31 “density fraction” from the separation process was pelleted and re-suspended a final time
32 in 2% sodium dodecyl sulfate (SDS) solution for one hour at room temperature with
33 gentle mixing to denature any proteins (7).

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38 *Protein gel electrophoresis:*

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40 Molecular weight standard “ladders” were run on both sides of all sample gel lanes to
41 correct for horizontal distortions commonly produced during gel electrophoresis. Protein
42 extractions were analyzed using two different molecular weight ladders and two different
43 staining methods, for comparison of sample protein size/weights and staining
44 efficiencies. One set of gels was reacted with a silver-based stain (Bio-Rad, Solano, CA,
45 USA), after the method of Gottlieb and Chavko (8), and Kaleidoscope (Bio-Rad) pre-

1 stained molecular weight ladders were used to calibrate the weight/size of protein bands.
2 These gels were imaged using a standard Epson scanner. The second set of gels was
3 stained with SyproOrange, a UV-fluorescent dye that specifically binds to proteins (9),
4 and protein bands were calibrated to broad-range SyproOrange molecular weight ladders.
5 These gels were imaged using a UV-VIS light gel documentation system (Bio-Rad).
6 Both SyproOrange and silver staining can be used to detect ≤ 10 ng of protein (Bio-Rad,
7 written communication).

8 9 *ZnS nanoparticle synthesis:*

10
11 Nano-ZnS was synthesized by the reaction of 150 mM ZnCl_2 and aqueous sulfide, which
12 were dissolved separately into 200 ml of ultra-pure (0.1 μm pore-size double-filtered
13 MilliQ; Millipore, Billerica, MA, USA) DI water and reacted drop wise under constant
14 stirring for 1.5 hr (4).

15 16 *ZnS nanoparticle/amino acid aggregation experiments:*

17
18 The initial size of ZnS precipitates was determined to be ≤ 3 nm diameter using a UV-vis
19 spectrometer (Ocean Optics, Dunedin, FL, USA) calibrated for ZnS particle size using
20 published TEM observations (10). The following experimental conditions were
21 established in separate glass flasks: (a) “blanks” (ultrapure water only), (b) ZnS
22 nanoparticles only, (c) a single amino acid only and (d) ZnS nanoparticles plus a single
23 amino acid. All incubation flasks were (in the following order) detergent-washed, rinsed
24 with MilliQ water three times, acid-washed in 0.1N HCl overnight, rinsed five times with
25 MilliQ water, and sealed with parafilm, prior to the experiment. After the addition of any
26 reagents, flasks were resealed with parafilm and kept sealed during the experiment
27 between sampling times. All flasks were gently stirred throughout the experiment by
28 acid-washed Teflon-coated magnetic stirrer bars. 1 ml aliquots from each experimental
29 condition were taken at 0, 0.5, 1, 3, 5, and 7 days of reaction progress. Between sample
30 measurements, 0.5 ml of 0.1 N HCl was flushed through the sample line and chamber,
31 followed by 3 ml of ultrapure water, to avoid cross-contamination of samples. Three
32 measurements of 40 – 60 accumulations each were obtained per analysis at each
33 sampling time point.

34 The amino acids chosen for these aggregation experiments represent the following
35 classes of structures and functional groups: alanine (nonpolar, aliphatic), aspartate
36 (acidic, negatively charged), cysteine (polar, uncharged, thiol-bearing), lysine (basic,
37 positively charged), phenylalanine (aromatic), proline (polar, uncharged, imine-bearing),
38 and serine (polar, uncharged, hydroxyl-bearing). Their conditional stability constants for
39 mono-ligand complexation of aqueous Zn^{2+} are given in Table 2, from data collected by
40 Martell and Smith (11).

41 42 *Dynamic light scattering analysis (DLS):*

43 DLS analysis was performed with a PD-Expert Workstation (Precision Detectors,
44 Bellingham, MA, USA) fitted with a quartz flow-through cell maintained at 25 °C. Laser

1 light (685 nm wavelength) scattered through 135° was sent via glass fiber to an optical
2 correlator. The DLS correlation function is a direct measurement of the diffusion
3 coefficient(s) of objects suspended in a solvent. For specific values of solvent viscosity
4 and refractive index, an effective hydrodynamic radius, R_H , of the object(s) can be
5 calculated (12). In the present case, we observed light scattering from aggregates of ZnS
6 nanoparticles. Because the exact shape and hydrodynamic properties of nanoparticulate
7 aggregates are not well known, there may be systematic differences between the
8 calculated R_H and spatial dimensions observed by alternative techniques (such as electron
9 microscopy). Determination of the distribution of ZnS aggregate sizes was performed
10 using proprietary Precision Detectors software (PrecisionDeconvolve) that employs a
11 regularization algorithm (13) to seek a smooth, non-negative size distribution function
12 that provides the best fit to the data. Low noise DLS data analyzed with this method can
13 resolve multimodal distributions, provided the diffusion coefficients differ by greater
14 than a factor of ~ 2.5 .

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19 **Supplementary Figure Captions:**

20

21 **Figure S1: Field-emission scanning electron microscopy (FESEM) secondary**
22 **electron image of micron-scale spheroidal aggregates of nanocrystalline biogenic**
23 **ZnS formed in the biofilm.** Scale bar is 2 μm .

24

25 **Figure S2: HRTEM image of cross-section through spheroidal biogenic ZnS**
26 **aggregate in the biofilm.** Multiple concentric rings (3-5 nm wide, shown by white
27 arrows) of low electron density attributed to porous regions separate bands of densely
28 aggregated nanoparticulate ZnS.

29

30 **Figure S3: High-resolution transmission electron microscopy (HRTEM) images of**
31 **biogenic ZnS nanoparticles formed in a biofilm of sulfate-reducing bacteria.** (A)
32 The smallest ZnS nanoparticles are crystalline, as shown by {111} lattice fringes in [02-
33 2] projection, and <2 nm-diameter. (B) Several aggregated nanoparticles with boundaries
34 indicated by dashed lines. (C) Disordered ZnS aggregates contain multiple stacking
35 faults (interpolated along pairs of white lines) that give rise to wurtzite subdomains
36 (across white line pairs) in sphalerite. (D) Example of aggregation producing faceted
37 surfaces and negative crystals (dashed white triangle). All scale bars are 2 nm.

38

39 **Figure S4: TEM image of biofilm organic matter, cells and ZnS aggregates.** Stained
40 ultramicrotomed sections of biofilm show abundant ZnS spheroidal aggregates (black
41 spheroidal features with strong diffraction contrast) surrounded by biofilm organic matter

1 (white arrows), and in close proximity to cells (black arrows). Dark grey spheroidal
2 features with lighter grey cores (grey arrows) are cells surrounded by capsular organic
3 matter that has been stained with uranyl acetate and lead citrate. Scale bars are 1 μ m.
4

5 **Figure S5: NanoSIMS secondary ion images showing ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S isotopic**
6 **distributions in ultramicrotomed TEM section of standard reference materials.** (A)
7 Composite isotope map of ^{12}C (blue), $^{12}\text{C}^{14}\text{N}$ (green), and ^{32}S (red) in ultramicrotomed
8 Balmat sphalerite. Secondary and tertiary colors vary in hue and intensity to reflect
9 relative proportions of two or three isotopic components, respectively. (B) Composite
10 isotope map of ultramicrotomed synthetic ZnS aggregates. $^{12}\text{C}^{14}\text{N}$ maximum counts are
11 \sim 200 in both (A) and (B), and are 2-3 orders of magnitude lower than the same intensity
12 in biofilm samples. (C) *Box* key to color-coded isotopic distributions seen in (A) and
13 (B). The key is projected through the ternary end member that would represent a
14 combination of maximum ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S counts (i.e. through “white”). Scale bars
15 =1 μ m.
16

17 **Figure S6: Microcentrifuge tubes of lithium-polytungstate solution (LST), biofilm**
18 **and ZnS.** The left-hand tube shows the color of pure LST. Homogenized biofilm is
19 introduced and subjected to low speed centrifugation to separate lower and higher density
20 fractions (middle tube). Density-driven separation is iteratively repeated until no further
21 separation is achieved (right-hand tube). Material at the top of the LST solution consists
22 predominantly of biofilm organic matter, while the pellet at the bottom is concentrated in
23 ZnS spheroids.
24

25 **Figure S7: NanoSIMS secondary ion images showing ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S isotopic**
26 **distributions in ultramicrotomed TEM section of biofilm and spheroidal ZnS**
27 **aggregates.** (A) Composite isotope map of ^{12}C (blue), $^{12}\text{C}^{14}\text{N}$ (green), and ^{32}S (red) in
28 biofilm area “D6.2”. Secondary and tertiary colors vary in hue and intensity to reflect
29 relative proportions of two or three isotopic components, respectively. (B) *Box* key to
30 color-coded isotopic distributions seen in (A) and (C). The key is projected through the
31 ternary end member that would represent a combination of maximum ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S
32 counts (i.e. through “white”). (C) TEM image of biofilm and ZnS spheroids in partial
33 field of view from (A). Scale bars =1 μ m.
34

35 **Figure S8: NanoSIMS secondary ion images showing ^{12}C , $^{12}\text{C}^{14}\text{N}$ and ^{32}S isotopic**
36 **distributions in ultramicrotomed TEM section of biofilm and spheroidal ZnS**
37 **aggregates.** (A) Composite isotope map of ^{12}C (blue), $^{12}\text{C}^{14}\text{N}$ (green), and ^{32}S (red) in
38 biofilm area “D6.3”. Secondary and tertiary colors vary in hue and intensity to reflect
39 relative proportions of two or three isotopic components, respectively. *Color wheel:* key
40 to isotopic distributions. (B) *Box* key to color-coded isotopic distributions seen in (A) and
41 (C). (C) TEM image of biofilm and ZnS spheroids in partial field of view from (A).
42 Scale bars =1 μ m.
43

44 **Figure S9: Dynamic light scattering (DLS) correlation functions acquired during**
45 **ZnS nanoparticle aggregation experiments: (A) 100 μ M cysteine, (B) 10 μ M ZnS, (C)**

1 10 μM ZnS and 100 μM cysteine. A trend in the data indicating continuous aggregation
2 was observed only for the sample containing both ZnS and cysteine. For this sample, the
3 scattering intensity increased (improving data quality) and there was a steady trend in the
4 correlation function threshold indicating the formation of larger suspended aggregates.
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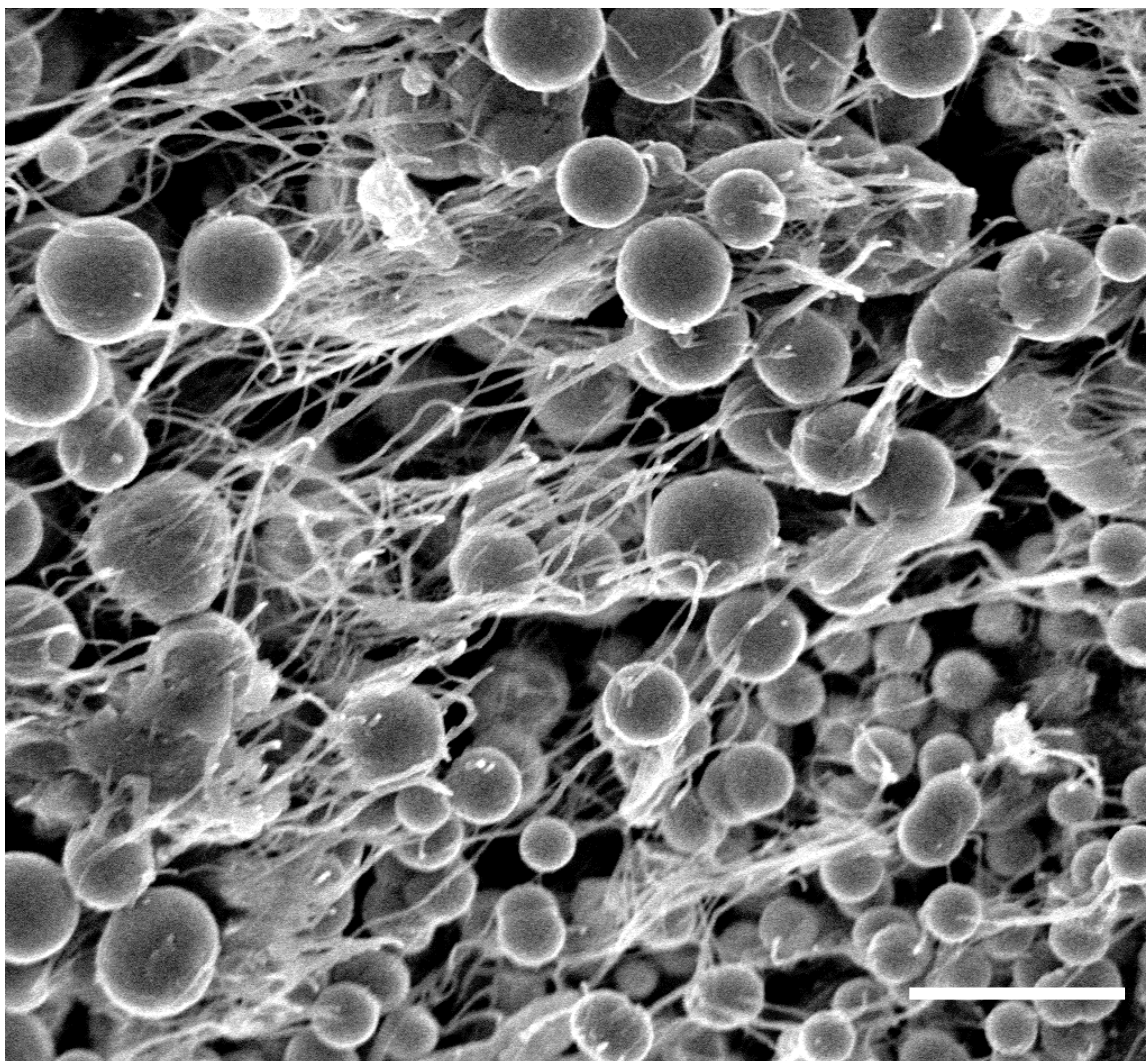
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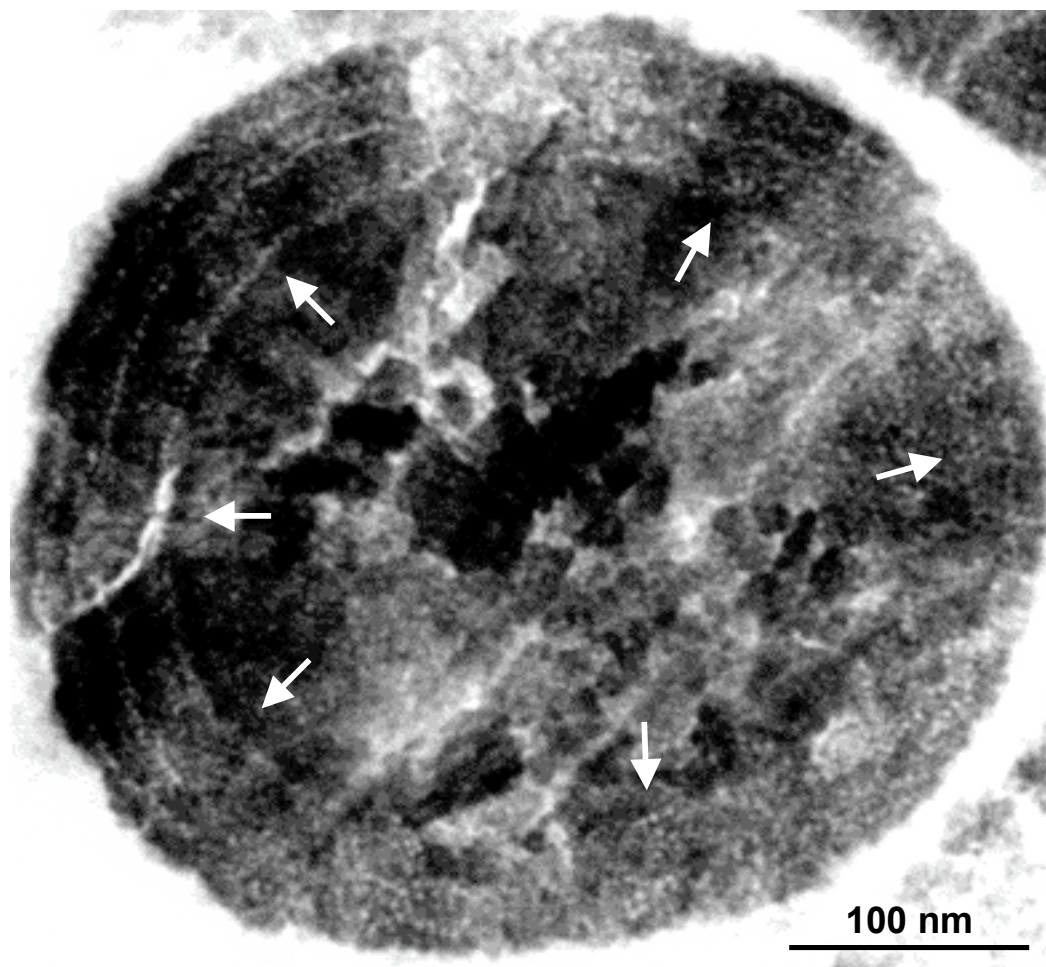
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9 **Figure S1**

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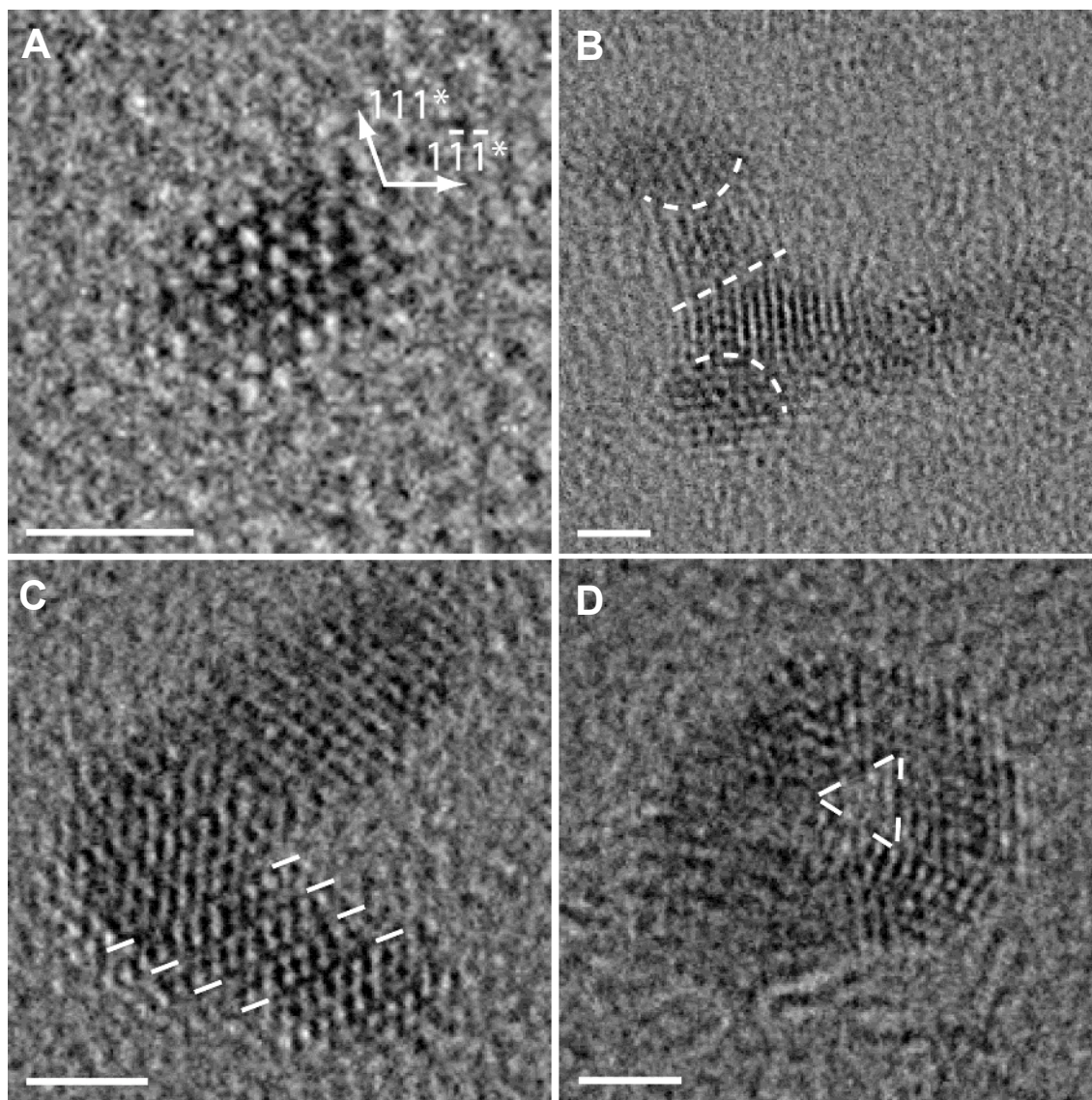
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1 **Figure S2**

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1 **Figure S3**

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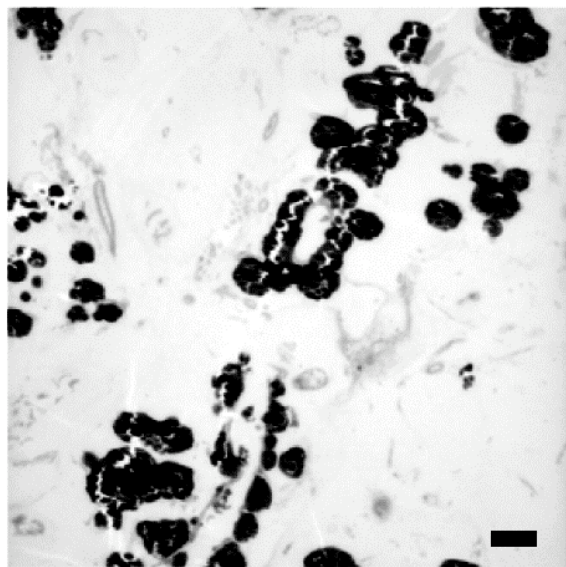
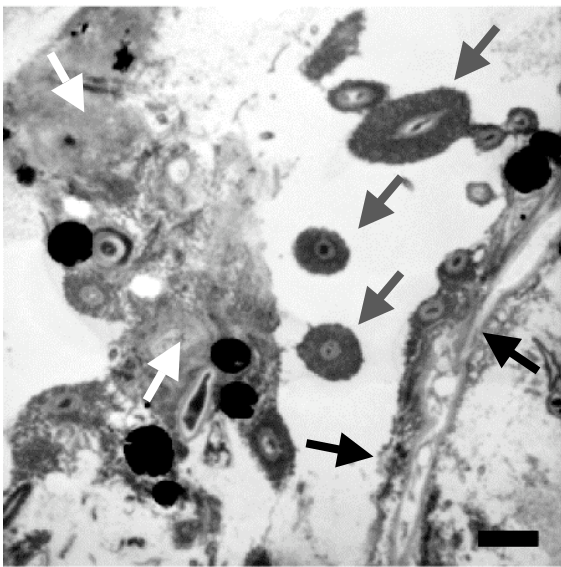
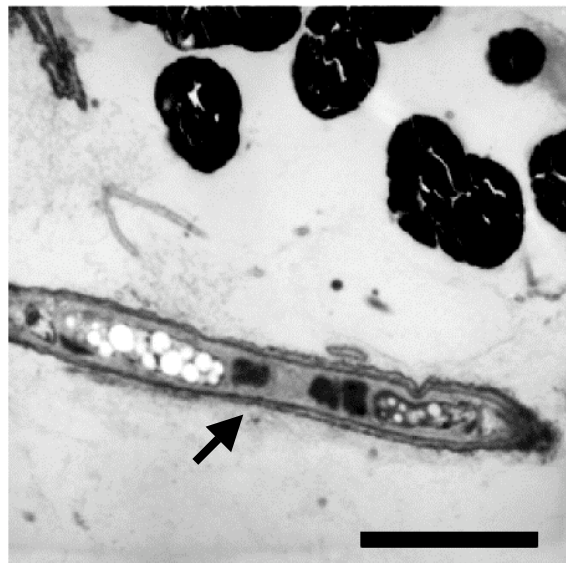
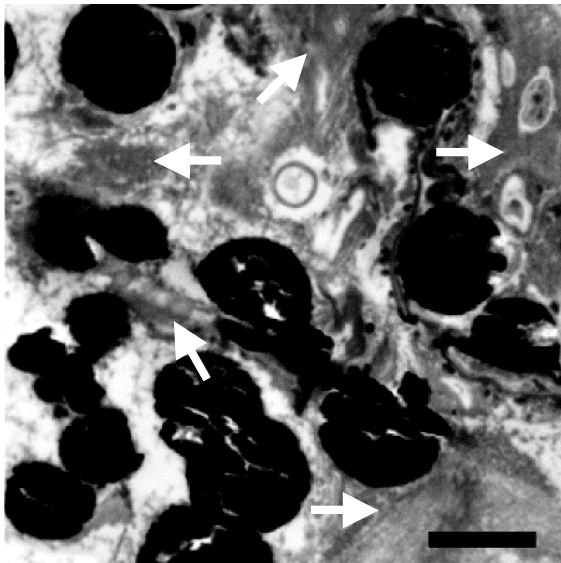
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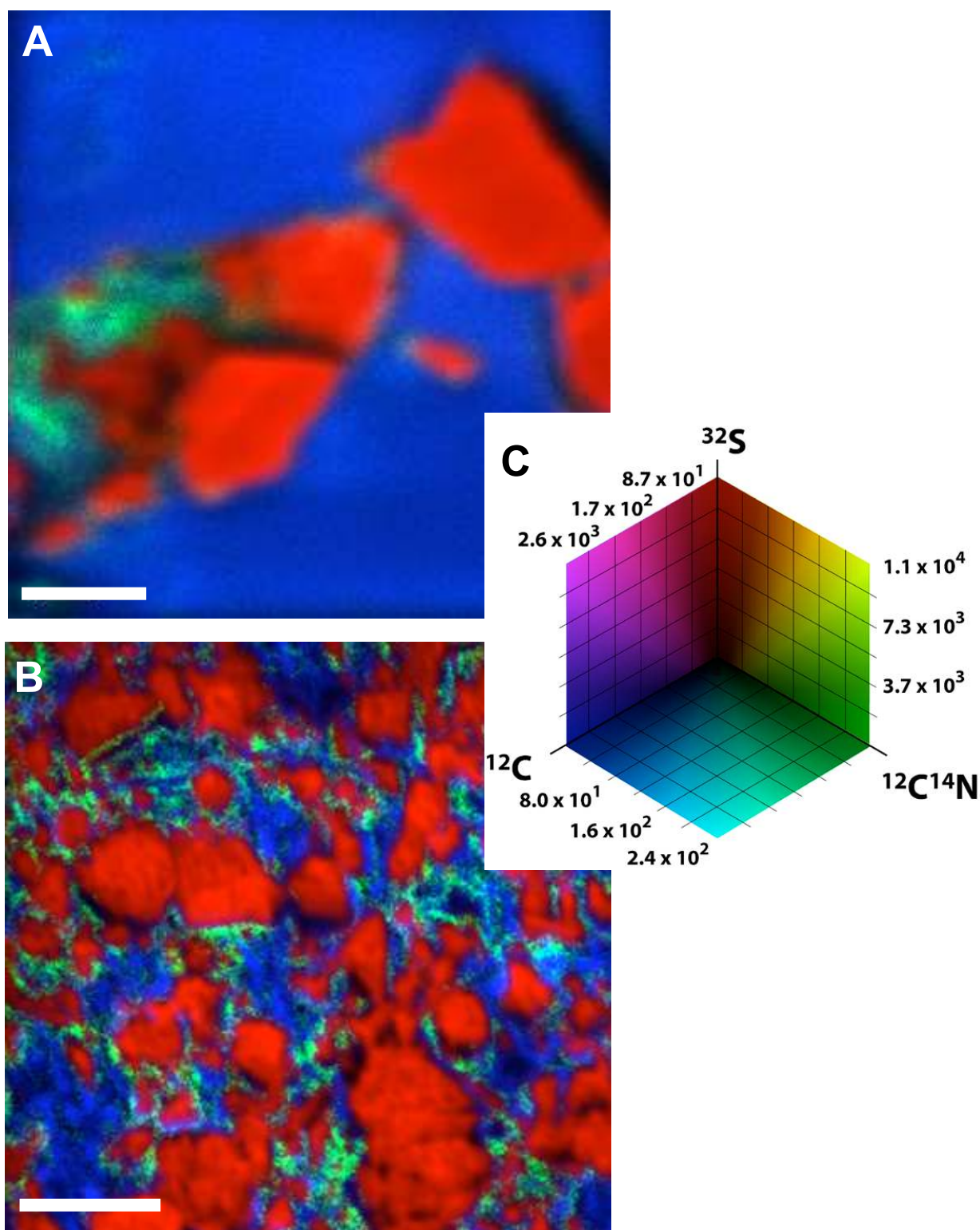
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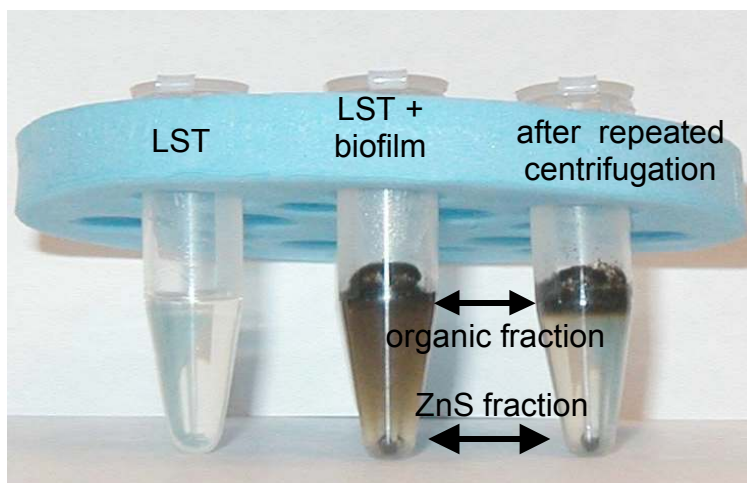
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1 **Figure S5**

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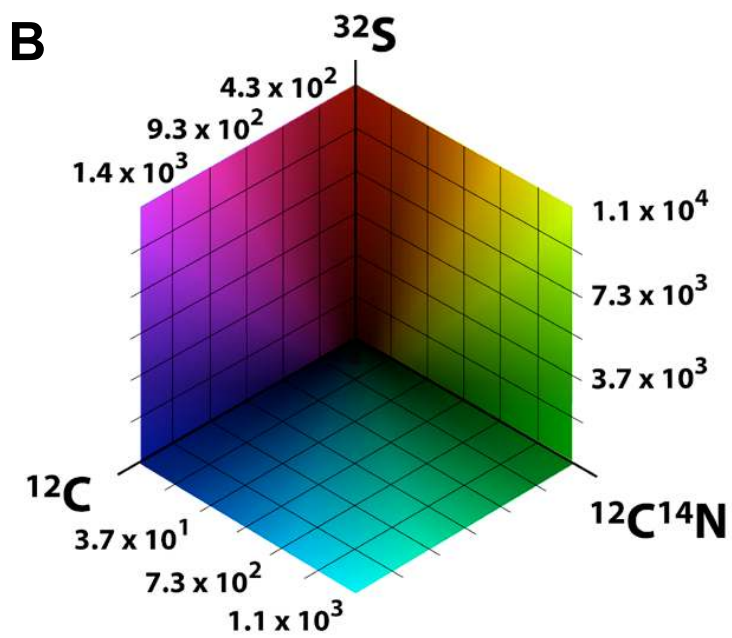
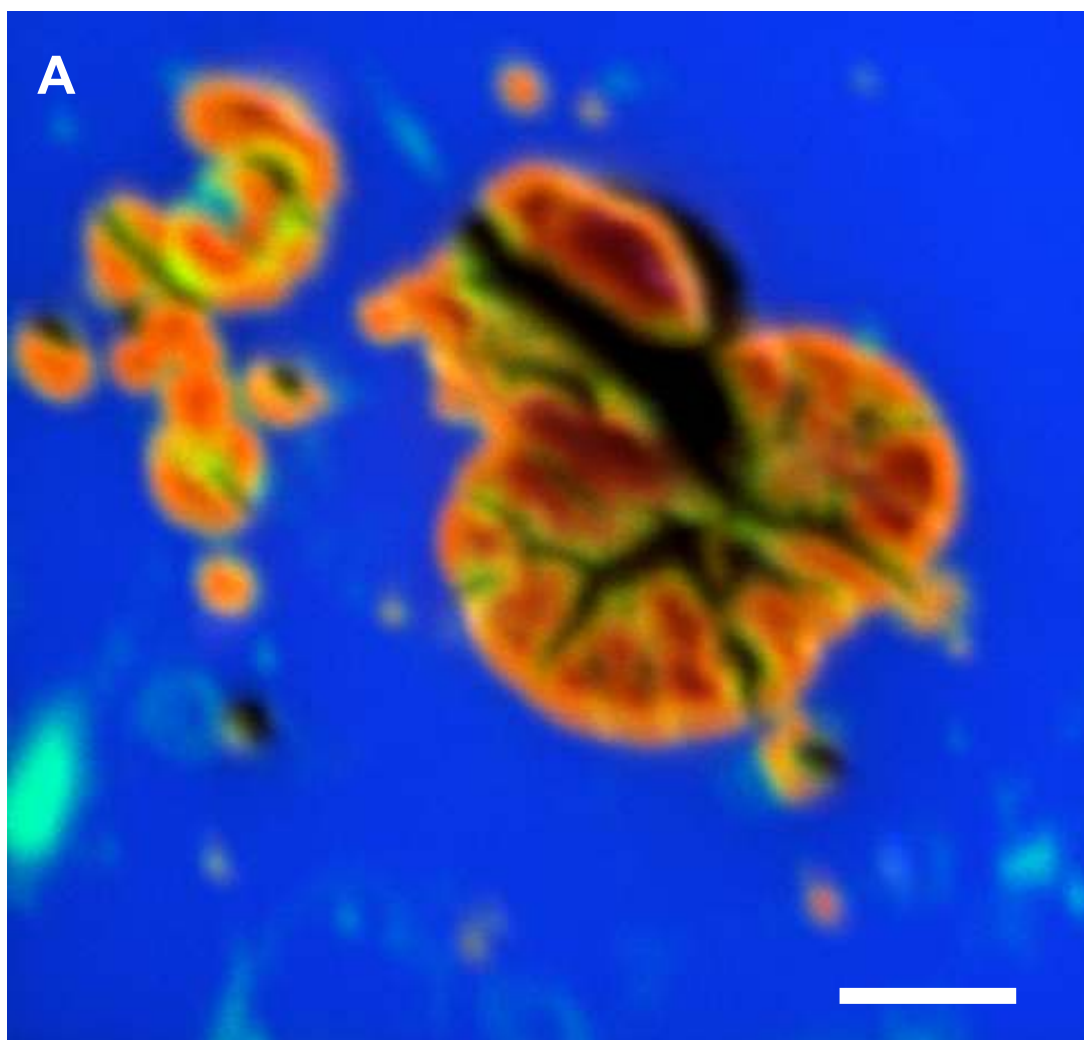
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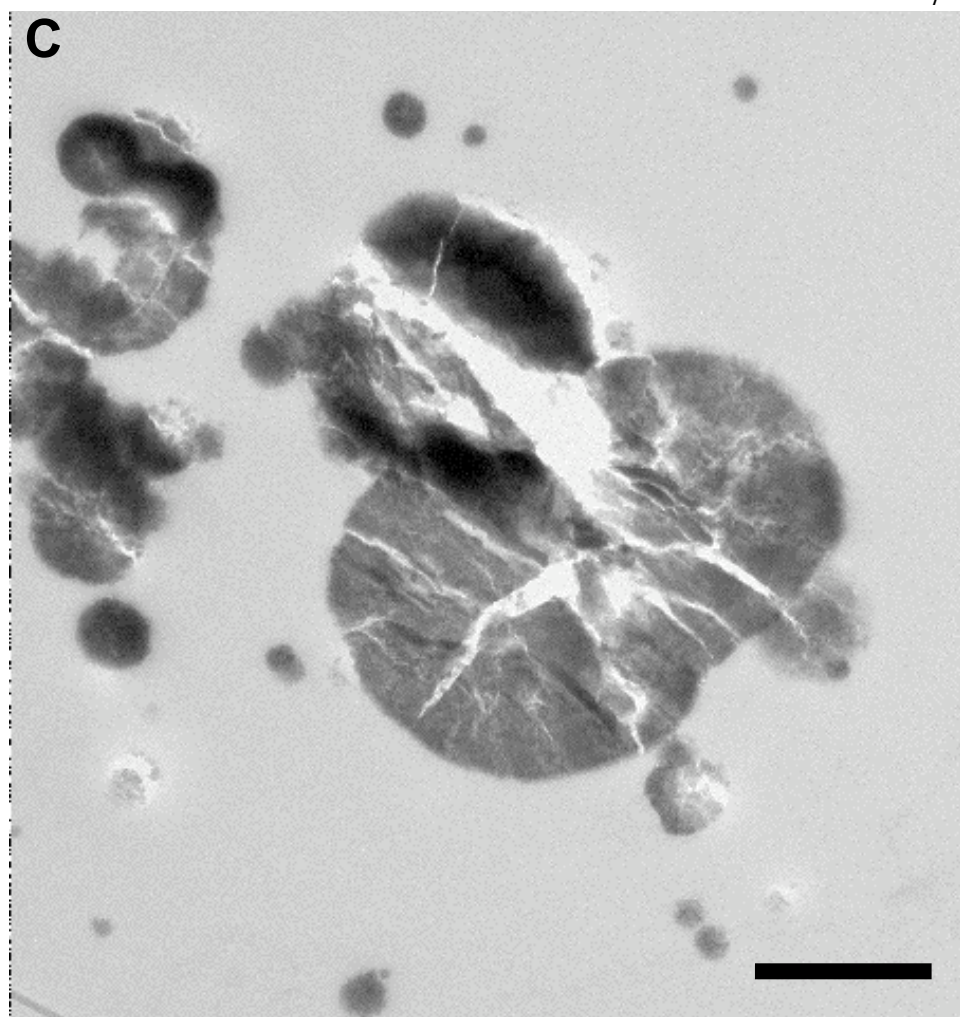
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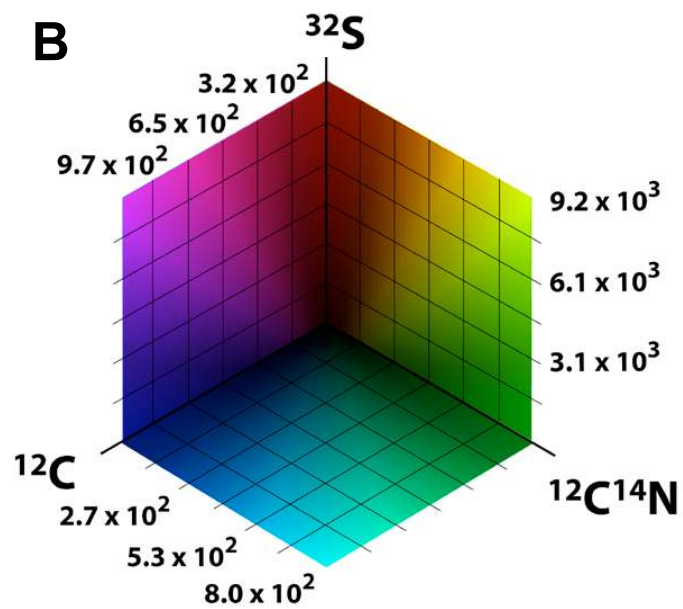
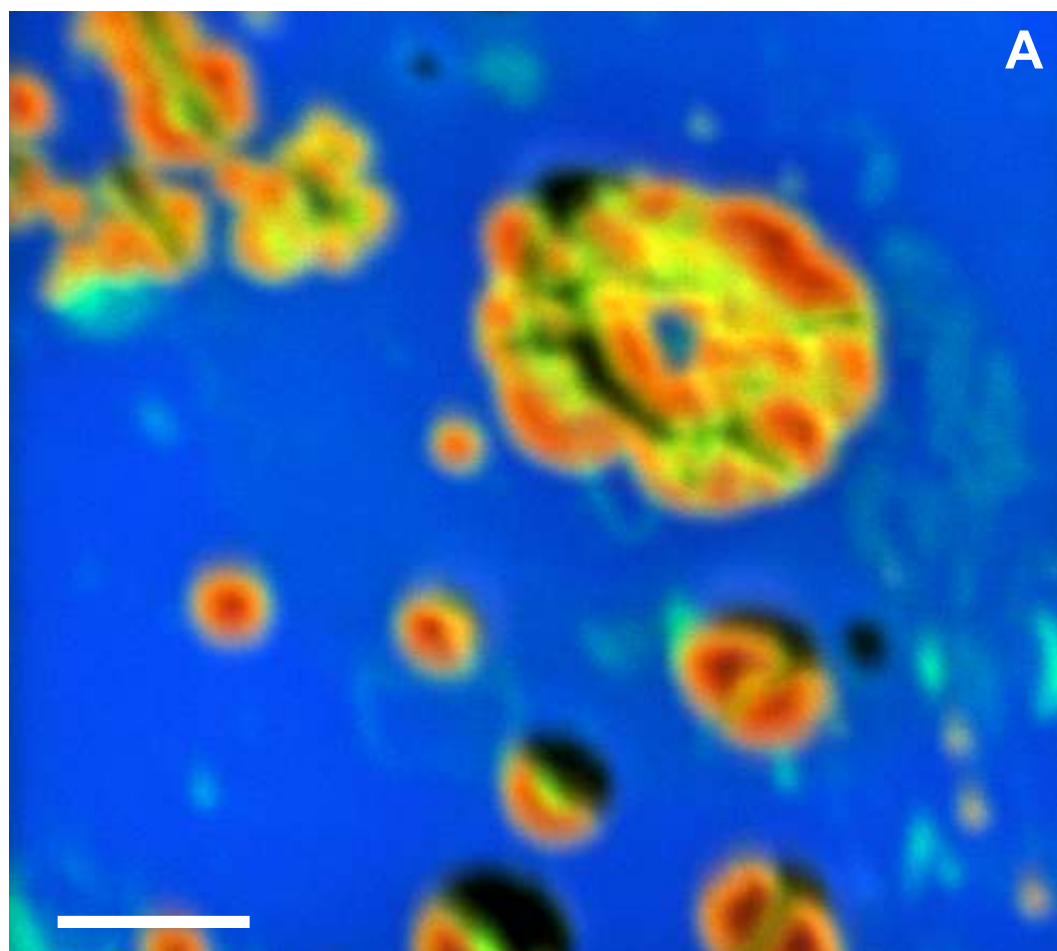
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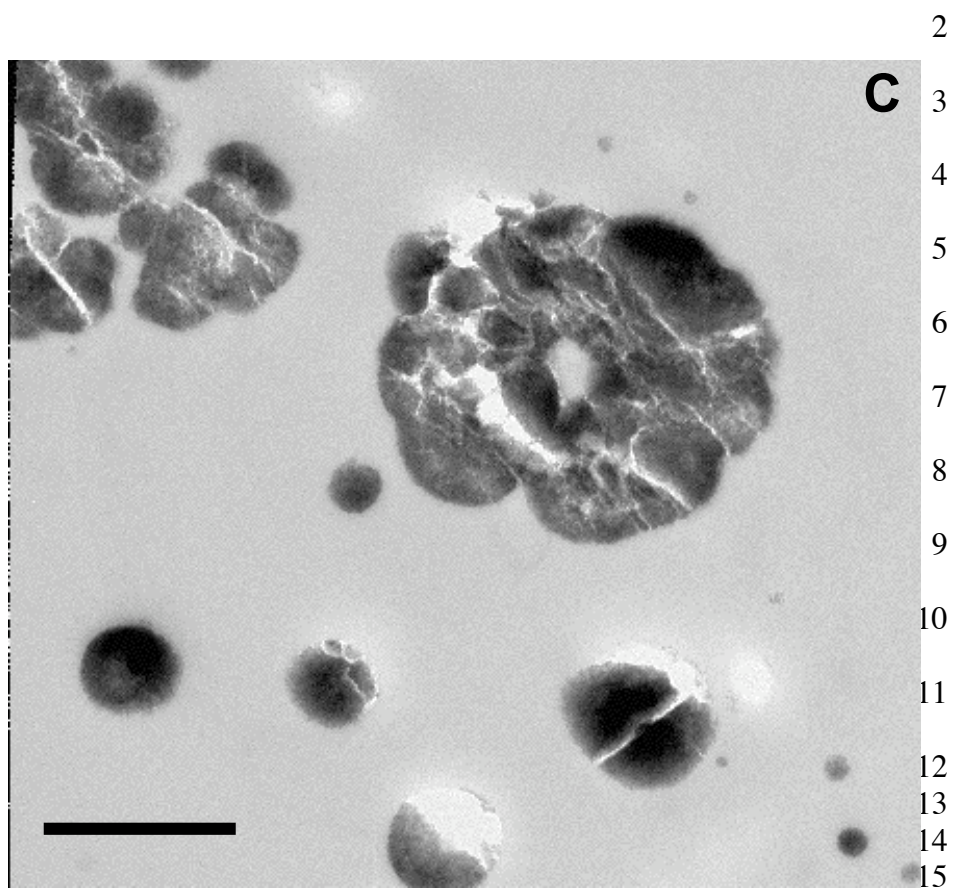
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1 **Figure S8**
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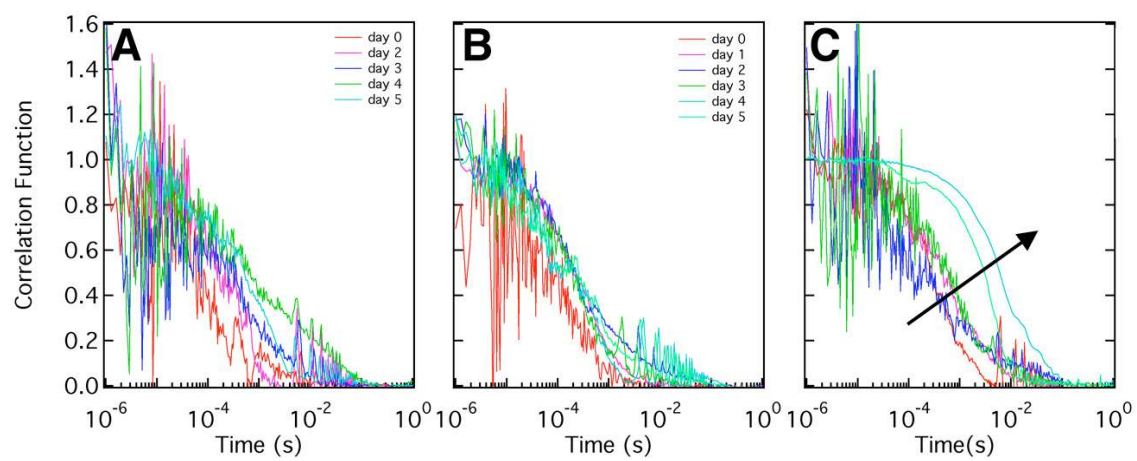


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1 **Figure S9**

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